IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF PROPOLIS (*APISMELLIFERA* LINN) ON CYCLOPHOSPHAMIDE TREATED IMMUNOSUPPRESSED RATS

A Dissertation submitted to

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Submitted by

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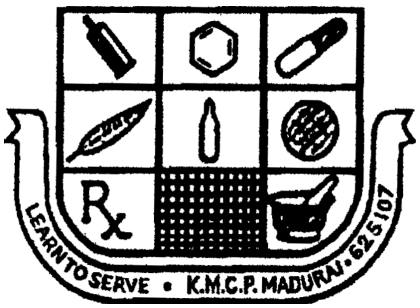

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CERTIFICATE

This is to certify that the dissertation entitled **"IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF PROPOLIS (*APISMELLIFERA* LINN) ON CYCLOPHOSPHAMIDE TREATED IMMUNOSUPPRESSED RATS"** submitted by **Mrs. S. PACKIALAKSHMI** in partial fulfillment for the degree of **"MASTER OF PHARMACY IN PHARMACOLOGY"** under The Tamilnadu Dr. M.G.R Medical University Chennai, at K.M. College of Pharmacy, Madurai–625107, is a bonafide work carried out by her under my guidance and supervision during the academic year of **2016 – 2017**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university.

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CONTENTS

INTRODUCTION

IMMUNITY

In biology, immunity is the balanced state of having adequate biological defenses to fight infection, disease, or other unwanted biological invasion, while having adequate tolerance to avoid allergy, and autoimmune diseases.

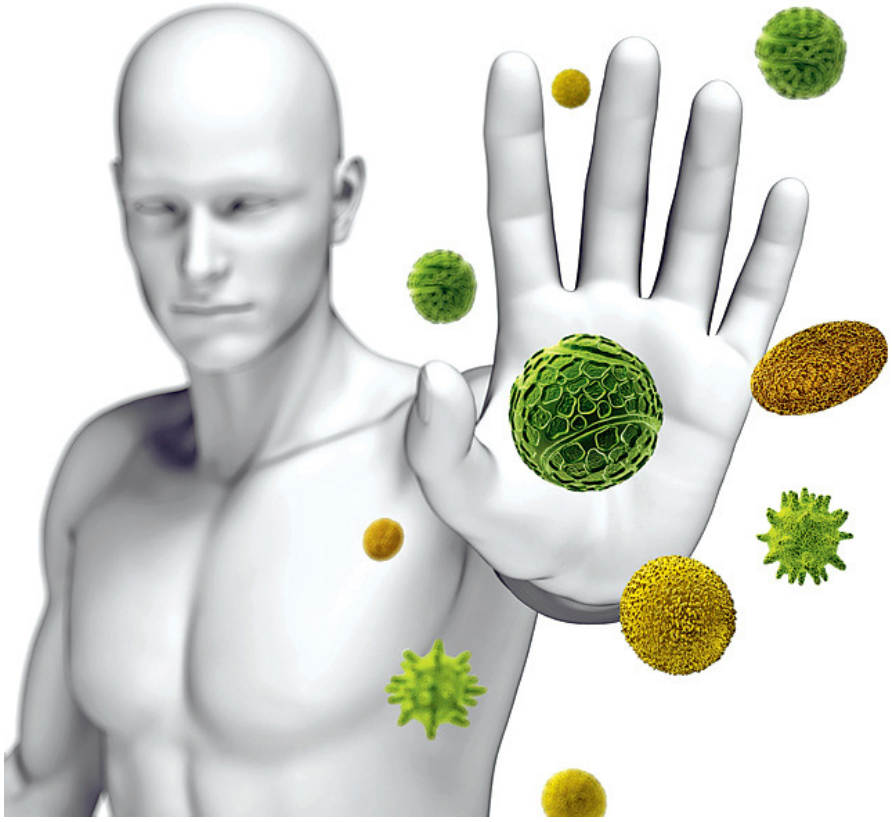
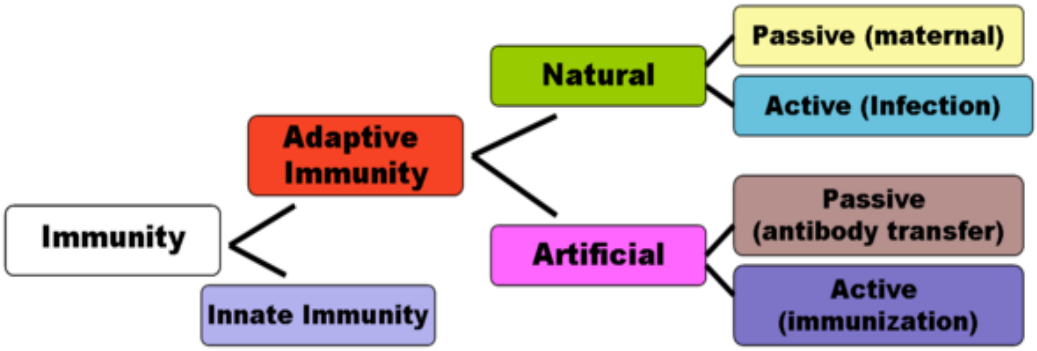


Fig. 1



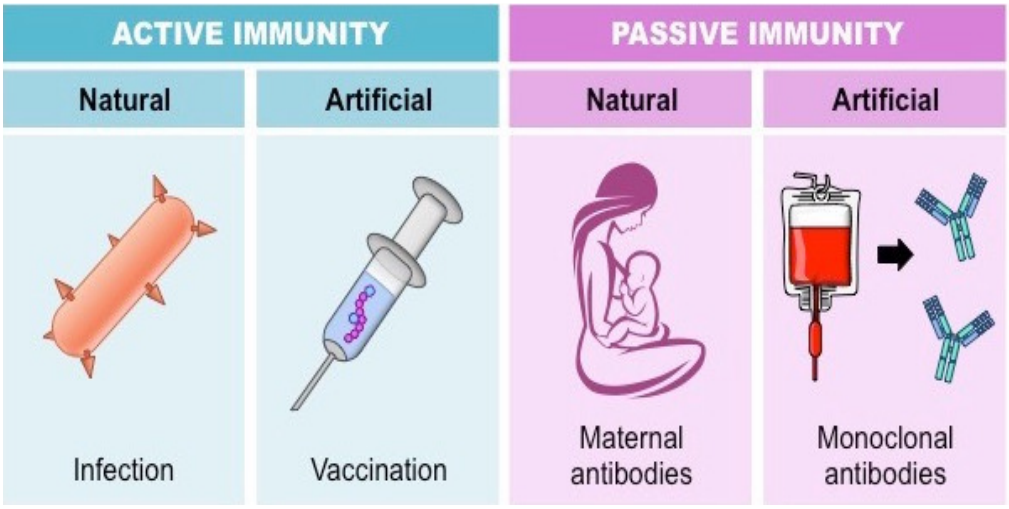


Fig. 2

Innate immunity, also called natural or native immunity. This immunity is by virtue of genetic constitutional make-up. It is there in the body without any external stimulation or a previous infection. It is divided into two types:- (a) Non-Specific innate immunity: A degree of natural resistance to all infections in general. (b) Specific innate immunity: This is a natural resistance to a particular kind of germ only. Some races or specific individual do not suffer from certain infectious diseases.

Adaptive immunity is often sub-divided into two major types depending on how the immunity was introduced. 'Naturally acquired immunity' occurs through contact with a disease causing agent, when the contact was not deliberate, whereas 'artificially acquired immunity' develops only through deliberate actions such as vaccination. Both naturally and artificially acquired immunity can be further subdivided depending on whether immunity is induced in the host or passively transferred from an immune host. 'Passive immunity' is acquired through transfer of antibodies or activated T-cells from an immune host, and is short lived—usually lasting only a few months—whereas 'active immunity' is induced in the host itself by antigen and lasts much longer, sometimes lifelong.

A further subdivision of adaptive immunity is characterized by the cells involved; humoral immunity is the aspect of immunity that is mediated by secreted antibodies, whereas the protection provided by cell mediated immunity involves T-lymphocytes alone. Humoral immunity is active when the organism generates its own antibodies, and passive when antibodies are transferred between individuals.

Similarly, cell mediated immunity is active when the organisms' own T-cells are stimulated and passive when T cells come from another organism.

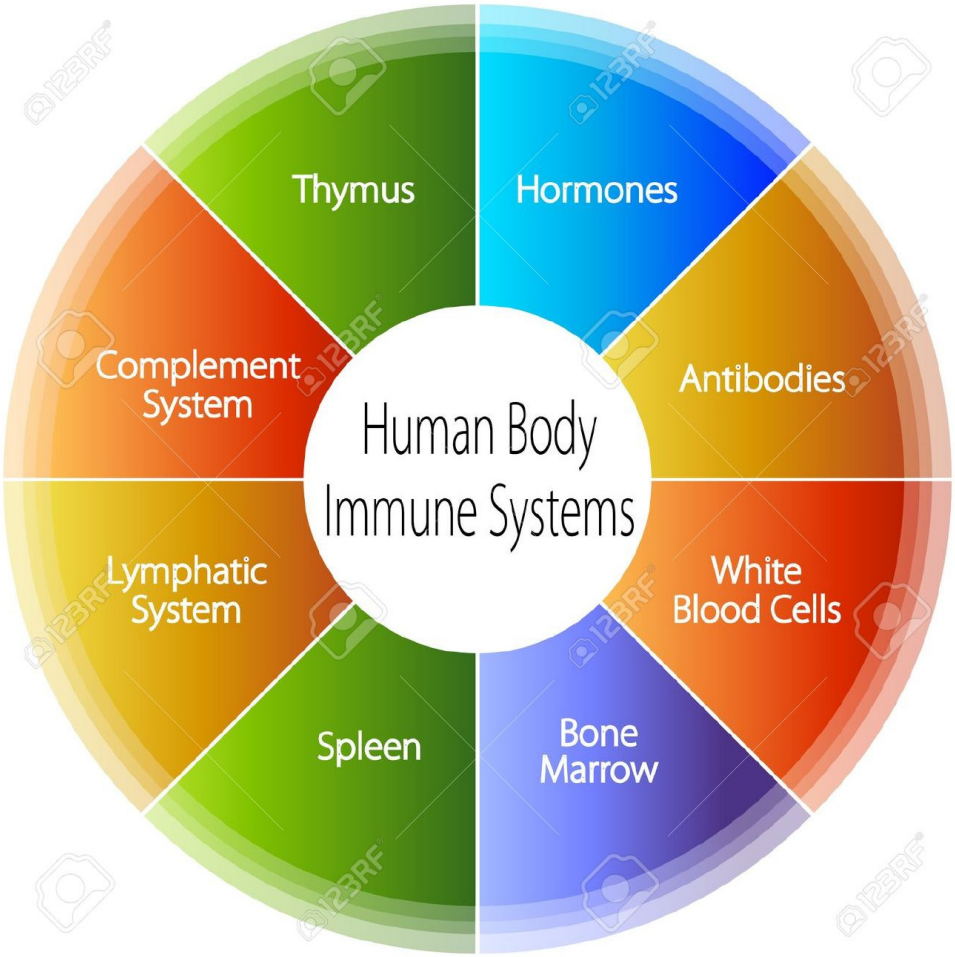


Fig. 3

Passive immunity is the transfer of active immunity, in the form of readymade antibodies, from one individual to another. Passive immunity can occur naturally, when maternal antibodies are transferred to the foetus through the placenta, and can also be induced artificially, when high levels of human (or horse) antibodies specific for a pathogen or toxin are transferred to non-immune individuals. Passive immunization is used when there is a high risk of infection and insufficient time for the body to develop its own immune response, or to reduce the symptoms of ongoing or immunosuppressive diseases[1].Passive immunity provides immediate protection, but the body does not develop memory, therefore the patient is at risk of being infected by the same pathogen later[2].

Maternal passive immunity is a type of naturally acquired passive immunity,

and refers to antibody-mediated immunity conveyed to a fetus by its mother during pregnancy. Maternal antibodies (MatAb) are passed through the placenta to the fetus by an FcRn receptor on placental cells. This occurs around the third month of gestation. IgG is the only antibody isotype that can pass through the placenta. Passive immunity is also provided through the transfer of IgA antibodies found in breast milk that are transferred to the gut of the infant, protecting against bacterial infections, until the newborn can synthesize its own antibodies.

Fig. 4 One of the first bottles of diphtheria antitoxin produced (Dated 1895)

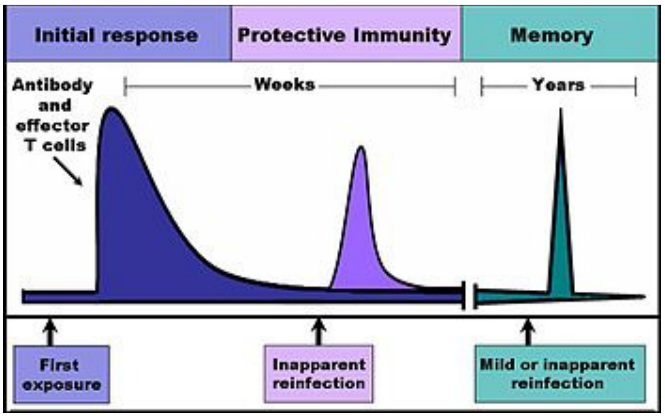
Artificially acquired passive immunity is a short-term immunization induced by the transfer of antibodies, which can be administered in several forms; as human or animal blood plasma, as pooled human immunoglobulin for intravenous (IVIG) or intramuscular (IG) use, and in the form of monoclonal antibodies (MAb). Passive transfer is used prophylactically in the case of immuno deficiency diseases, such as hypogammaglobulinemia[3]. It is also used in the treatment of several types of acute infection, and to treat poisoning. Immunity derived from passive immunization lasts for only a short period of time, and there is also a potential risk for hypersensitivity reactions, and serum sickness, especially from gamma globulin of non-human origin.

The artificial induction of passive immunity has been used for over a century to treat infectious disease, and prior to the advent of antibiotics, was often the only specific treatment for certain infections. Immunoglobulin therapy continued to be a first line therapy in the treatment of severe respiratory diseases until the 1930s, even after sulfonamide lot antibiotics were introduced.

Passive or "adoptive transfer" of cell-mediated immunity, is conferred by the transfer of "sensitized" or activated T-cells from one individual into another. It is rarely used in humans because it requires histocompatible (matched) donors, which

are often difficult to find. In unmatched donors this type of transfer carries severe risks of graft versus host disease. It has, however, been used to treat certain diseases including some types of cancer and immunodeficiency. This type of transfer differs from a bone marrow transplant, in which (undifferentiated) hematopoietic stem cells are transferred.

Active immunity



The time course of an immune response

Due to the formation of immunological memory, reinfection at later time points leads to a rapid increase in antibody production and effector T cell activity. These later infections can be mild or even unapparent.

When B cells and T cells are activated by a pathogen, memory B-cells and T-cells develop, and the primary immune response results. Throughout the lifetime of an animal these memory cells will "remember" each specific pathogen encountered, and are able to mount a strong secondary response, if the pathogen is detected again. The primary and secondary responses were first described in 1921 by English immunologist Alexander Glenny[4] although the mechanism involved was not discovered until later.This type of immunity is both active and adaptive because the body's immune system prepares itself for future challenges. Active immunity often involves both the cell-mediated and humoral aspects of immunity as well as input from the innate immune system.

Naturally acquired active immunity occurs when a person is exposed to a live pathogen, and develops a primary immune response, which leads to immunological memory. This type of immunity is "natural" because it is not induced by deliberate exposure. Many disorders of immune system function can affect the formation of

active immunity such as immuno deficiency (both acquired and congenital forms) and immunosuppression.

Artificially acquired active immunity can be induced by a vaccine, a substance that contains antigen. A vaccine stimulates a primary response against the antigen without causing symptoms of the disease. The term vaccination was coined by Richard Dunning, a colleague of Edward Jenner, and adapted by Louis Pasteur for his pioneering work in vaccination. The method Pasteur used entailed treating the infectious agents for those diseases so they lost the ability to cause serious disease. Pasteur adopted the name vaccine as a generic term in honor of Jenner's discovery, which Pasteur's work built upon.

Fig. 5 Poster from before the 1979 eradication of smallpox, promoting vaccination

In 1807, Bavaria became the first group to require that their military recruits be vaccinated against smallpox, as the spread of smallpox was linked to combat[5]. Subsequently the practice of vaccination would increase with the spread of war.

There are four types of traditional vaccines:[6]

- Inactivated vaccines are composed of micro-organisms that have been killed with chemicals and/or heat and are no longer infectious. Examples are vaccines against flu, cholera, plague, and hepatitis A. Most vaccines of this type are likely to require booster shots.

- Live, attenuated vaccines are composed of micro-organisms that have been cultivated under conditions which disable their ability to induce disease. These responses are more durable and do not generally require booster shots. Examples include yellow fever, measles, rubella, and mumps.
- Toxoids are inactivated toxic compounds from micro-organisms in cases where these (rather than the micro-organism itself) cause illness, used prior to an encounter with the toxin of the micro-organism. Examples of toxoid-based vaccines include tetanus and diphtheria.
- Subunit vaccines are composed of small fragments of disease causing organisms. A characteristic example is the subunit vaccine against Hepatitis B virus.

Most vaccines are given by hypodermic or intramuscular injection as they are not absorbed reliably through the gut. Live attenuated polio and some typhoid and cholera vaccines are given orally in order to produce immunity based in the bowel.

Immuno suppression is a reduction of the activation or efficacy of the immune system. Some portions of the immune system itself have immunosuppressive effects on other parts of the immune system, and immune suppression may occur as an adverse reaction to treatment of other conditions.

In general, deliberately induced immune suppression is performed to prevent the body from rejecting an organ transplant, treating graft-versus-host disease after a bone marrow transplant, or for the treatment of auto-immune diseases such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, or Crohn's disease. This is typically done using medications, but may involve surgery (spleen removal), plasmapharesis, or radiation.

A person who is undergoing immune suppression, or whose immune system is weak for other reasons (for example, chemotherapy or HIV), is said to be immuno compromised. An immune suppressant is any agent that weakens the immune system, including immunosuppressive drugs and some environmental toxins.

Fig. 6 Micrograph showing an opportunistic infection due to immunosuppression - large (blue) cell below-center-left infected with a polyomavirus. Urine cytology specimen.

Immunosuppressive drug

Administration of immunosuppressive medications or immunosuppressants is the main method of deliberately induced immunosuppression. In optimal circumstances, immunosuppressive drugs are targeted only at any hyperactive component of the immune system, and in ideal circumstances would not cause any significant immunodeficiency. However, in essence, all immunosuppressive drugs have the potential to cause immunodeficiency. Immunodeficiency can cause increased susceptibility to opportunistic infections and decreased cancer immunosurveillance. The term immunotoxin is also sometimes used (incorrectly) to label undesirable immunosuppressants, such as various pollutants. Immunosuppressants may be prescribed when a normal immune response is undesirable, such as in autoimmune diseases.

Cortisone was the first immunosuppressant identified, but its wide-ranging side-effects limited its use. The more specific azathioprine was identified in 1959, but it was the discovery of ciclosporin in 1970 that allowed significant expansion of kidney transplantation to less well-matched donor-recipient pairs as well as broad application to liver transplantation, lung transplantation, pancreas transplantation, and

heart transplantation. After organ transplantation, the body will nearly always reject the new organ(s) due to differences in human leukocyte antigen haplotypes between the donor and recipient. As a result, the immune system detects the new tissue as "foreign", and attempts to remove it by attacking it with recipient white blood cells, resulting in the death of the donated tissue. Immunosuppressants are given as an attempt to prevent this rejection; the side-effect is that the body becomes more vulnerable to infections and malignancy, as in advanced HIV infection.] At the same time, people with previous cancer who require immune suppression are not more likely to have a recurrence[7].

Immunodeficiency

Non-deliberate immune suppression can occur in, for example, malnutrition, aging, many types of cancer (such as leukemia, lymphoma, multiple myeloma), and certain chronic infections such as Human Immunodeficiency virus (HIV)[8]. The unwanted effect in non-deliberate immune suppression is immunodeficiency that results in increased susceptibility to pathogens such as bacteria, viruses, or fungi.

Immunodeficiency is also a potential adverse effect of many immunosuppressant drugs. In this sense, the scope of the term immune suppression in general includes both beneficial and potential adverse effects of decreasing the function of the immune system, whereas the term immunodeficiency in general refers solely to the adverse effect of increased risk for infection.

Immunosuppressants in Organ Transplantation

What are immunosuppressants?

Immunosuppressants are a class of drugs that suppress the immune response through various mechanisms. In organ transplantation, immune suppressants are used to prevent the body from either recognition or attacking the foreign organ via various immune responses. They should only be used under the supervision of appropriately trained and experienced staff. The types of drugs that use for immune suppression in organ transplant are:

- Calcineurin inhibitors (cyclosporin, tacrolimus)
- Corticosteroids (eg: methylprednisolone, dexamethasone, prednisolone)
- Cytotoxic immunosuppressants (azathioprine, chlorambucil, cyclo-phosphamide, mercaptopurine, methotrexate)

- Immunosuppressant antibodies (eg: antithymocyte globulins, basiliximab, infliximab)
- Sirolimus derivatives (everolimus, sirolimus)
- Other immunosuppressants (mycophenolate)

Immune suppressants used for:

- Preventing organ rejection and reverse acute rejection in organ transplantation.
- Prevent and treat graft-versus-host disease.
- Minimise destruction of affected tissues in autoimmune and inflammatory diseases.

Drug treatment

Transplant rejection involves the body producing T and B cell and immune responses that recognise markers on foreign tissue called antigens. Treatment regimens used to prevent rejection employ drugs from different classes taking advantage of their complementary actions and minimising toxicity. Drug choice depends on the organ being transplanted and is tailored for each individual to minimise transplant-related morbidity.

Double drug treatment – usually a calcineurin inhibitor such as (Tacrolimus) or (cyclosporine) with either Imuran (azathioprine) or CellCept (mycophenolate).

Triple drug treatment – usually a calcineurin inhibitor such as (Tacrolimus) or (cyclosporine), a corticosteroid and either (azathioprine) or (mycophenolate).

Quadruple drug treatment – as for triple drug treatment plus an induction course with an immunosuppressant antibody (antithymocyte globulin, basiliximab or daclizumab).

Immunosuppression for organ transplants usually involves triple or quadruple drug treatment. The intensity of immune suppression is initially high but tends to be reduced to a maintenance level that is determined by individual factors and the type of organ transplant.

Initial Treatment

Initially a specialist may give a corticosteroid and either (azathioprine) or mycophenolate (sometimes with a calcineurin inhibitors such as (tacrolimus) or (cyclosporin) for initial immune suppression immediately before cadaveric donor

transplantation, or for several days before planned live donor transplantation.

Mechanism of immune suppressants:

Calcineurin inhibitors

Calcineurin inhibitors react in the body to block the activity of calcineurin. This results in controlling the body's immune response and reducing the body recognising and attacking the foreign organ.

Neoral (cyclosporin) is available as a microemulsion, which has greatly enhanced its oral bioavailability, with much less variation in absorption within and between patients.

Evidence suggests that (tacrolimus) may be slightly more efficacious than (cyclosporin) and they are now used in equal numbers of patients worldwide. Absorption is hindered by food, so usually it needs to be taken on an empty stomach.

Sirolimus is a recently developed immunosuppressant, which is very similar to Prograf (tacrolimus). It has many adverse effects, but has much less renal toxicity than calcineurin inhibitors, and is currently mainly used in lung transplantation to 'rescue' patients from chronic renal failure, by substituting it for (cyclosporin).

Cell cycle inhibitors

Imuran (azathioprine) is used in 60% of patients and mycophenolate mofetil in 40%. These drugs stop the production of cells called B and T cell that cause the immune response.

Corticosteroids

Corticosteroids are thought to reduce the synthesis and secretion of a variety of inflammatory mediators.

Side effects of immunosuppressants

The side effects for each type of medication vary but the most common side effects that occur to less than 10% of patients are:

- Alopecia
- Dyspepsia
- Increased susceptibility to infections (eg: oral, vaginal and intertriginous candidiasis)

- Masking of signs of infection
- Increased appetite
- Delayed wound healing

Some of the rare serious side effects that can occur in less than 1% are:

- Muscle weakness and wasting (particularly symptomatic on drug withdrawal)
- Amenorrhoea
- Psychosis
- Euphoria
- Depression
- Hirsutism
- Gingival hyperplasia

Immunosuppressants and malignancy

Immunosuppression increases the chance of getting skin cancer so take additional measures to protect the skin from the sun such as wearing protective clothing, wearing sunscreen and avoiding exposure to the sun for prolonged periods of time.

Overview of immunosuppression in liver transplantation

Due to advances in immunosuppression and improvements in surgical techniques, liver transplantation has become an extremely successful treatment option for patients with end-stage liver disease, with one-year graft survival rates exceeding 80%[9]. Currently, there are eight patients worldwide who have survived more than three decades after liver transplantation[10].

Organ transplantation initially came to light with the first successful kidney transplantation in 1954 on monozygotic twins; however, immunosuppression was limited to total body irradiation which was largely fatal[11,12]. With the invention of 6-mercaptopurine (6-MP) and azathioprine (AZA) in the 1950s along with the introduction of corticosteroids as combination therapy by Starzl in the 1960s, there was noticeable improvement in kidney allograft survival, although one-year survival still did not exceed 50%. Multiple interventions including splenectomy, thymectomy and thoracic duct drainage were employed with minimal success.

The first successful human liver transplant was performed by Thomas Starzl in Denver in 1967 on an 18-month-old child with unresectable hepatoblastoma[10]. The immunosuppressive regimen included anti-lymphocyte globulin (ALG), AZA and prednisolone and the child survived for more than a year.

However, the next significant breakthrough in immunosuppression did not occur until the discovery of cyclosporine (CYA) in 1972 from the soil fungus Tolypocladiuminflatum. Borel et al[13] first described its remarkable immunosuppressive properties in 1976 and by the 1980s there was international affirmation of its effectiveness. CYA quickly became the standard of care for maintenance immunosuppression in solid organ transplant recipients. This paved the way for the current era of liver transplantation, which has since continued to evolve with the discovery of multiple novel immunosuppressive agents.

IMMUNOSUPPRESSION

Effective immunosuppression in transplantation relies on preventing the immune system from rejecting the allograft while preserving immunologic control of infection and neoplasia. Although the mechanism is not completely understood, transplanted livers rarely reject compared to other organs, do not require an HLA-matched donor, and may offer a protective effect for other simultaneously transplanted organs[14,15]. Both micro- and macrochimerism models have been used to explain this phenomenon, as well as that of hepatic dissolution of donor specific antibodies.

Ideally, the long-term objective is to achieve immune tolerance or the ability to alter the recipient's immune system in order to promote long-term graft function without immunosuppressive therapy, while maintaining immunity to infectious agents[16]. Unfortunately, except for a small minority of patients (approximately 20%) who have been successfully weaned off immunosuppressive medications, most experience immunologic rejection with the discontinuation of these drugs and have to be maintained on at least low doses of these medications[17-21].

Immunosuppressive regimens include calcineurin inhibitors, anti-metabolites, mTOR inhibitors, steroids and antibody-based therapies. These agents target different sites in the T cell activation cascade, usually by inhibiting T cell activation or proliferation or via T cell depletion. The selection of agents is based on an

individual's medical history as well as on institution experience and preference. Most immunosuppressive regimens combine drugs with different sites of action of T cell response, allowing for dosage adjustments to minimize side effects and toxicities. Currently, the mainstay of maintenance immunosuppressive regimens are calcineurin inhibitors (CNIs), used in greater than 95% of transplant centers upon discharge, although there is a known increased risk of renal impairment[22,23], metabolic derangements, neurotoxicity and de novo malignancies[24] with the long-term use of these medications.

Calcineurin Inhibitors

CYA and tacrolimus are the two CNIs approved for use in organ transplantation and are the principal immune suppressives used for maintenance therapy. The routine use of these medications in liver transplant recipients has dramatically decreased the incidence of rejection and graft loss. The primary mode of action is inhibition of T cell activation. CYA binds to cyclophilin which results in inhibition of the calcium/calmodulin-dependent phosphatase, calcineurin. The binding to cyclophilin interferes with calcineurin's de-phosphorylation of nuclear factor of activated T cells (NFAT), preventing translocation of NFAT into the nucleus and up-regulation of pro-inflammatory cytokines. The end result is the inhibition of IL-2 gene transcription and T cell activation and proliferation. Tacrolimus also inhibits calcineurin but binds specifically to FK506-binding protein (FKBP-12).

The immunosuppressive effects of the CNIs are related to total drug exposure which can be estimated by measuring blood 12-h troughs. The potency of tacrolimus is estimated to be 100 times greater on a molar level when compared to CYA. Although several earlier studies showed tacrolimus to be superior to CYA in the prevention of cellular rejection[25-27], another more recent multi-center trial showed no significant differences between the two medications with regard to acute rejection episodes, death or graft loss[28]. Both CNIs are metabolized principally by the cytochrome P450 system and therefore have significant interactions with multiple medications requiring careful monitoring of drug levels

Table No. 1

COMMON SIDE EFFECTS OF IMMUNOSUPPRESSIVE AGENTS

DRUG	ADVERSE EFFECTS
Tacrolimus	Nephrotoxicity, neurotoxicity[1], diabetes[1], hyperkalemia, metabolic acidosis, hypertension, hyperlipidemia
Cyclosporine	Nephrotoxicity, neurotoxicity, diabetes, hyperlipidemia[1], hypertension[1], hyperkalemia, metabolic acidosis, gingival hyperplasia, hypertrichosis
MMF	Myelosuppression, gastrointestinal side effects, viral infections (CMV, HSV), spontaneous abortions in pregnant women
Sirolimus	Hyperlipidemia, myelosuppression, proteinuria, poor wound healing, pneumonitis, skin rash
Corticosteroids	Diabetes, hypertension, obesity, osteoporosis, avascular necrosis, growth retardation, Cushingoid features, psychosis, poor wound healing, adrenal suppression, cataracts

Antimetabolites

Both mycophenolatemofetil (MMF) and mycophenolate sodium (MPS) undergo immediate first-pass metabolism in the liver into the active compound mycophenolic acid (MPA), which was first discovered in 1893[29]. However, the immunosuppressive properties of MPA were not recognized until the 1990s. MPA inhibits inosine-5'-monophosphate dehydrogenase (IMPDH)[30], the rate-limiting

enzyme in the de novo synthesis of guanosine nucleotides. Inhibition of the IMPDH pathway results in selective blockade of lymphocyte proliferation[31].

The major advantage in using the MPAs is their lack of renal toxicity. In patients with pre-existing renal disease, they have been used in conjunction with low-dose CNIs as part of a renal-sparing protocol with promising results[32,33]. Ideally, these medications should be initiated when renal dysfunction is first noted, although emerging data suggests the benefits of MPAs in reversing long-standing renal disease due to its association with decreased TGF-β levels[34-36]. MPAs are rarely used as monotherapy in transplant recipients given their higher rates of rejection compared to the CNIs[37,38], although more recent data demonstrate the safety of this approach when carried out carefully[39,40]. However, in patients previously on CNIs or mTOR inhibitors with evidence of acute rejection, MPAs are often added as supplemental immunosuppressive therapy.

Azathioprine is another antimetabolite which was predominantly used for the prevention of rejection in the 1960s but has since been largely replaced by the MPAs. It is selectively used in a few centers in combination with other immunosuppressive medications, primarily CNIs and steroids.

mTOR Inhibitors

The two mTOR inhibitors approved for organ transplantation are sirolimus (SRL) and everolimus (EVL), although neither has been approved for use in liver transplantation to date. They bind intracellularly to FK506 binding protein (FKBP12) but unlike tacrolimus, they do not inhibit calcineurin activity. Rather, the complex is a highly specific inhibitor of mammalian target of rapamycin complex 1 (mTORC1)[41] which has a direct effect on the cell signaling pathway required for cell cycle progression. This subsequently inhibits IL-2 signaling to T cells, thus preventing T cell proliferation. Similar to the CNIs, sirolimus is metabolized by the cytochrome P450 system and requires therapeutic drug monitoring (Table11).

The first reported study illustrating the effectiveness of sirolimusmonotherapy for maintenance of immunosuppression in liver transplantation was in 1999 by Watson et al[42]. However, two subsequent large studies examining sirolimus de novo therapy with tacrolimus and corticosteroids were terminated early due to excess hepatic artery thrombosis (HAT). As a result, sirolimus carries a black box label

warning which cautions against the possible development of early post-transplant HAT. Subsequent studies have since disputed this finding[43-45] however, mTOR inhibitors are rarely used as de novo therapy.

Importantly, in patients with CNI-induced nephrotoxicity, conversion to sirolimus therapy has proved to be effective with ensuing improvements in renal function[46-48]. Again, sirolimus conversion should be initiated early since late conversion rarely improves chronic renal dysfunction[49]. In fact, several studies have shown that in patients with pre-existing renal disease, sirolimus can worsen nephrotoxicity and promote proteinuria[50-52].

Recent studies have also shown potential anti-tumor properties of sirolimus[53-56] which might be of importance in patients undergoing liver transplantation for HCC. Zimmerman et al[57] examined the role of sirolimus-based maintenance therapy in post-transplant recipients with a history of HCC and found that overall survival was increased in the sirolimus arm compared to the CNI arm. Clinical trials examining the anti-cancer effects of mTOR inhibitors in liver transplant recipients with HCC have been encouraging[58] and new trials are ongoing.

Antibody-Based Therapies

Polyclonal antibodies

Polyclonal antibodies, including anti-thymocyte (ATG) and anti-lymphocyte globulins (ALG), have been used since the early days of liver transplantation and are prepared by inoculating rabbits or horses with human lymphocytes or thymocytes. Their mechanism of action is rapid lymphocyte depletion due to complement-mediated cell lysis and uptake by the reticuloendothelial system (RES) of opsonized T cells[59]. In addition, they may also cause partial T cell activation and blockade of T cell proliferation[60]. Polyclonal antibodies were routinely used as induction therapy in liver transplantation along with corticosteroids and AZA before discovery ofCYA.

Lymphocyte depletion is believed to play a role in preparing the recipient's immune system to adapt and recognize the transplanted organ as self and prevent destruction of the allograft. Accordingly, studies have shown that ATG administration results in regulatory T cell (Treg) expansion in vitro and in vivo[61-63]. Tregs or suppressor T cells are responsible for preventing activation of the immune system and maintaining tolerance to self-antigens.

Currently, approximately 20% of transplant centers use these agents for induction purposes[64] and recent data support the administration of thymoglobulin induction to delay CNI use and avoid renal toxicity without increasing the risk of rejection or HCV recurrence[65-67]. A few studies have also successfully shown the benefit of using these medications as induction therapy to avoid post-transplant corticosteroid use[68,69] without an increased incidence of acute rejection. This is especially important in HCV recipients where high-dose pulsed corticosteroid therapy can significantly accelerate liver fibrosis. At present, anti-lymphocyte antibodies are used extensively to treat steroid-resistant acute rejection and are successful in 70%-96% of patients[70-72].

Monoclonal antibodies

Monoclonal antibodies include the anti-IL-2 receptor (CD25) antibodies, anti-CD52 antibody and muromonab-CD3 (OKT3). The two anti-IL-2 receptor antibodies approved for clinical use are basiliximab (Simulect), a chimeric protein, and daclizumab (Zenapax), a humanized protein. Both antibodies are specific for the α chain of the IL-2 receptor, CD25, which is only expressed on activated T cells. These antibodies remain in the circulatory system for weeks after initiation of therapy and have been used successfully with low-dose CNIs in preventing acute rejection in the early post-transplant period[73-75]. They also have fewer side effects compared to the anti-lymphocyte globulins, rarely cause the typical first-dose infusion reactions and are associated with less risk of opportunistic infections and PTLD.

Muromonab-CD3 (OKT3) targets the CD3 molecule on T cells and causes depletion of lymphocytes by massive T cell lysis[76] and cytokine release[77]. This profound cytokine release can lead to pulmonary edema and acute respiratory distress and rarely, intra-graft thrombosis and aseptic meningitis[78,79]. As a result, antihistamines and intravenous steroids are routinely used as pre-medication to reduce this "cytokine release syndrome". Several days after OKT3 administration, T lymphocytes no longer express CD3 and are considered to be immunologically incompetent[80]. OKT3 is primarily used in liver transplantation for steroid-resistant acute rejection[81,82] and has a success rate of complete recovery in 50% of patients. OKT3 use should be limited in the HCV population as several studies have confirmed exacerbation of disease recurrence with this agent[83,84].

PROPOLIS

Propolis or bee glue is a resinous mixture that honey bees produce by mixing saliva and beeswax with exudate gathered from tree buds, sap flows, or other botanical sources. It is used as a sealant for unwanted open spaces in the hive. Propolis is used for small gaps (approximately 6 millimeters (0.24 in) or less), while larger spaces are usually filled with beeswax. Its color varies depending on its botanical source, the most common being dark brown. Propolis is sticky at and above room temperature, 20°C (68°F). At lower temperatures, it becomes hard and very brittle.

Fig. 7 Resins in hive

The composition of propolis varies from hive to hive, from district to district, and from season to season[85]. Normally, it is dark brown in color, but it can be found in green, red, black, and white hues, depending on the sources of resin found in the particular hive area. Honey bees are opportunists, gathering what they need from available sources, and detailed analyses show that the chemical composition of propolis varies considerably from region to region, along with the vegetation. In northern temperate climates, for example, bees collect resins from trees, such as poplars and conifers (the biological role of resin in trees is to seal wounds and defend against bacteria, fungi and insects). "Typical" northern temperate propolis has approximately 50 constituents, primarily resins and vegetable balsams (50%), waxes (30%), essential oils (10%), and pollen (5%). Propolis also contains persistent lipophilic acaricides, a natural pesticide that deters mite infestations[86].

In neotropical regions, in addition to a large variety of trees, bees may also gather resin from flowers in the genera Clusia and Dalechampia, which are the only known plant genera that produce floral resins to attract pollinators[87]. Clusia resin contains polyprenylated benzophenones[88-90]. In some areas of Chile, propolis contains

viscidone, a terpene from Baccharis shrubs[91], and in Brazil, naphthoquinone epoxide has recently been isolated from red propolis[92], and prenylated acids such as 4-hydroxy-3,5-diprenyl cinnamic acid have been documented[93]. An analysis of propolis from Henan, China found sinapinic acid, isoferulic acid, caffeic acid, and chrysin, with the first three compounds demonstrating antibacterial properties[94]. Also, Brazilian red propolis, largely derived from Dalbergia ecastaphyllum plant resin, has high relative percentages of the isoflavonoids 3-hydroxy-8,9-dimethoxypterocarpan and medicarpin[95]. Other flavonoids commonly present include galangin and pinocembrin[96]. Caffeic acid phenethyl ester (CAPE) is also a component of some varieties of propolis from New Zealand[97].

Medical uses

Propolis has been used in traditional medicines for thousands of years[98,99]. The National Institutes of Health rates propolis as "possibly effective" for treating cold sores, genital herpes, and post-surgery mouth pain. Propolis is also used to make cough drops for cough and throat irritation[100]. Currently, there is "insufficient evidence" to rate the effectiveness of propolis in treating other conditions[101].

Biomedical research

Propolis is being researched for the potential development of new drugs focusing on a variety of its properties, including those for possible immunomodulatory, anti-diabetic and anti-ulcer applications[102].

Beneficial effects of propolis on human health and neurological diseases

Propolis is a natural product, collected by honeybees Apis mellifera, from various plant sources. Propolis is extensively used in foods and beverages because it improves human health. It contains more than 300 natural compounds such as polyphenols, phenolic aldehydes, sequiterpene-quinones, coumarins, amino acids, steroids and inorganic compounds. Propolis exhibits a broad spectrum of biological and pharmacological properties such as antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, antitumor, anticancer, antiulcer, hepatoprotective, cardio-protective, and neuroprotective actions. The chemical composition and beneficial properties of propolis vary greatly depending on the phytogeographical areas, seasonal collection time, and botanical source. Polyphenols found in fruits and vegetables are beginning to receive increased attention due to their vital role in

protecting neural cells from oxidative stress and neuroinflammation associated with normal aging and chronic age-related diseases. Propolis is one of the most abundant sources of polyphenols (mainly flavonoids and phenolic acids). This overview is an attempt to discuss the molecular mechanism underlying the potential beneficial effects of propolis on human health and neurological diseases.

Propolis is used for canker sores and infections caused by bacteria (including tuberculosis), by viruses (including flu, H1N1 "swine" flu, and the common cold), by fungus, and by single-celled organisms called protozoans. Propolis is also used for cancer of the nose and throat; for boosting the immune system; and for treating gastrointestinal (GI) problems including Helicobacter pylori infection in peptic ulcer disease. Propolis is also used as an antioxidant and anti-inflammatory agent.

People sometimes apply propolis directly to the skin for wound cleansing, genital herpes and cold sores; as a mouth rinse for speeding healing following oral surgery; and for the treatment of minor burns.Propolis seems to have activity against bacteria, viruses, and fungi. It might also have anti-inflammatory effects and help skin heal.

Propolis is possibly safe when taken by mouth or applied to the skin appropriately. It can cause allergic reactions, particularly in people who are allergic to bees or bee products. Lozenges containing propolis can cause irritation and mouth ulcers.

Pregnancy and breast-feeding:

There is not enough reliable information about the safety of taking propolis if you are pregnant or breast-feeding. Stay on the safe side and avoid use.

Asthma:

Some experts believe certain chemicals in propolis may make asthma worse. Avoid using propolis if you have asthma.

Bleeding conditions:

A certain chemical in propolis might slow blood clotting. Taking propolis might increase the risk of bleeding in people with bleeding disorders.

Allergies:

Do not use propolis if you are allergic to bee by-products including honey, conifers, poplars, Peru balsam, and salicylates.

Surgery:

A certain chemical in propolis might slow blood clotting. Taking propolis might increase the risk of bleeding during and after surgery. Stop taking propolis 2 weeks before surgery.

Medications that slow blood clotting (Anticoagulant / Antiplatelet drugs)

Propolis might slow blood clotting and increase bleeding time. Taking propolis along with medications that also slow clotting might increase the chances of bruising and bleeding. Some medications that slow blood clotting include aspirin, clopidogrel (Plavix), dalteparin (Fragmin), enoxaparin (Lovenox), heparin, ticlopidine (Ticlid), warfarin (Coumadin), and others.

Propolis might increase the amount of time it takes for blood to clot. Taking it along with other herbs and supplements that slow blood clotting can slow blood clotting even more and could increase the risk of bleeding and bruising in some people. Some of these herbs include angelica, clove, danshen, garlic, ginger, ginkgo, Panax ginseng, and others.

Applied to the skin:

- For cold sores: A 3% propolis ointment (Herstat or ColdSore-FX) applied 5 times daily.
- For herpes outbreak: A 3% propolis ointment (Herstat or ColdSore-FX) applied to the blisters 4 times daily.
- As a mouth rinse after mouth surgery: A solution containing propolis, water, and alcohol.

Other names

Acide de Cire d'Abeille, Baume de Propolis, Bee Glue, Bee Propolis, Beeswax Acid, Cire d'Abeille Synthétique, Cire de Propolis, Colle d'Abeille, Hive Dross, Pénicilline Russe, Propóleos, Propolis Balsam, Propolis Cera, Propolis d'Abeille, Propolis Resin, Propolis Wax, Résine de Propolis, Russian Penicillin, Synthetic Beeswax.

Methodology

To learn more about how this article was written, please see the Natural Medicines Comprehensive Database methodology.

Fig. 8

As an Antioxidant, bee propolis neutralizes free radicals that would otherwise damage molecules and lead to cell damage known to cause degenerative conditions.

As an Antimicrobial agent, bee propolis was shown in a test for anti-microbial action to diminish the growth of various bacteria, yeast, and fungus responsible for common ailments such as dental caries, vaginal yeast infections, salmonella, and stomach ulcers.

In addition, scientific trials and studies also suggest bee propolis can accelerate wound healing in diabetes, promote healthy cells, reduce outbreaks from mouth ulcers (RAC), and reduce inflammation associated with various conditions.

REVIEW OF LITERATURE

Propolis is the bee product with the highest antimicrobial activity. The antibacterial activity of propolis has been confirmed by numerous scientific studies. Antibacterial activity has been demonstrated against both gram positive and gram-positive, both aerobic and anaerobic types. Although the composition of propolis differs considerably depending on its botanical origin, all examined types of propolis revealed a strong antibacterial activity[103,104].

The antibacterial activity of poplar propolis and other types of propolis of different geographical and botanical origin was similar[105].

Poplar propolis gathered by *Apis mellifera caucasica* had a higher antibacterial activity than the one gathered by *Apis mellifera anatolica* and *Apis mellifera carnica*[106]

More recent research has revealed antibacterial activity against *Micrococcus luteus, Salmonella typhimurium*[107]*Klesbsiella pneumonae*[108]. In recent study, it has been shown that propolis has a stong antibacterial activity against[109] different plant pathogens[110] propolis is also based on quorum sensing inhibitory (QSI) action, the flavonoid pinocembrin being an important QSI agent[111].

Generally, biologically activity decreases with increasing storage. However it was found that propolis solution in ethanol stored for 10-15 years results not in result in a decrease, but in an increase of antibacterial activity[112].

Antifungal activity

Poplar propolis is the bee product with the highest antifungal activity as tested with 40 yeast strains of *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Trichosporon spp*[113].

Antivirus activity

Propolis kills the fungi and also the viruses, while the growth of the latter is also inhibited[114]. Propolis acts against many different viruses. Most notable is its activity against the influenza virus, found in propolis of different origin[115] and in Brazilian green propolis296 CAPE, a poplar propolis constituent is a prominent antiviral substance[116].

Antioxidant activity

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules and so to prevent such changes. The antioxidant effect correlates roughly with the anti-inflammatory and hepatoprotective activity.

Although the phenolic content seems to vary according to the botanical origin, antioxidant effects for most propolis types have been reported.

Compared to pollen and royal jelly, propolis extracts exhibited the highest antioxidant activity[117].

Hepatoprotective activity and anti-radiation activity

The liver is perhaps the hardest working organ of the body. It has hundreds of tasks to perform, including detoxification of the blood. A sluggish liver means fatigue and toxemia and a high risk of various chronic diseases. Phenolics are known to have a hepatoprotective function. Hepatoproctive activity for different types ofpropolis has been reported, which correlated to the antioxidant activity[118-120]. Propolis counteracts hepatoxic effects of alcohol liver injury in mice and also of paracetamol induced liver damage of mice111 and carbon tetrachloride induced liver damages in rats[121].

The anti-radiation effect of propolis have been reviewed by Orsolic in 2010. As an antioxidant propolis has a powerful effect to counteract radiation as tested in tumor cells or animals. Propolis act also in apoptosis (cell death) of cancer cells thus improving the anti-cancer effect of radiation[122].

Propolis supplementation is prophylactic for liver health and for counteracting the damaging effect of tumor irradiation.

Immunomodulating effects

The immunomodulating effect has been reviewed in 2007 by Sforcin[123]. All propolis types have immune stimulating activity. However, the active substances of the various types of propolis are different.

Action on microphages

In vitro and in vivo assays demonstrated the modulatory action of propolis on murine peritoneal macrophages,increasing their microbicidal activity and stimulating the lytic activity of natural killer cells against tumor cellsby enhancing antibody

production. The best immunostimulating results were observed when propolis was administered over a short-term to animals. Both poplar and baccharis propolis increase the microphage activity[123].

Action on lymphocytes and antibody production

Both poplar and baccharis propolis can have an immunostimulalting effect by increasing antibody production and by activating B and T lymphocytes, an adjuvant like activity of propolis. The propolis compoundschrysine, quercetin, and galangin have a antiparasitic activity[124].

Propolis can be regarded as a supplement for the stimulation of the immune system.

Antitumor effects

The antitumor activity of propolis has been reviewed Orsolic, 2010, shows that the chemopreventive activity of propolis in animal models and cell cultures are likelyto be the result of their ability to inhibit DNA synthesis in tumour cells, their capability to induce apoptosis (cell death) of tumour cells, and their property to activate macrophages to produce factors capable of regulating thefunction of B-, T- and NK-cells, respectively. Especially interesting is the synergy between propolis and anticancer agents. Moreover, flavonoids from propolis play a protective role against the toxicity of the chemotherapeutic agents or radiation in mice, giving hope that they may have similar protective action in humans. The combination with an adjuvant antioxidant therapy may enhance the effectiveness of chemotherapy by ameliorating the side effect on leukocytes, liver and kidneys and consequently enabling dose escalation.

Many polyphenols have an anti-metastatic activity, caffeic acid phenethyl ester (CAPE) from poplar propolis and Artepillin C from baccharis propolis has been identified as the most potent antitumor agents[125]. But antitumor effects of chrysin (poplar propolis) and both nemorosone and plukenetione A (inCuban propolis) have been reported. Regular consumption of propolis food supplements can have a preventive effect against mutation linked cancers in humans[126].

Propolis can be regarded as a supplement for cancer prevention.

PLAN OF WORK

Propolis, sometimes also called “bee glue”, is a strongly adhesive, resinous substance that honeybees collect from various plants, transformed and used by bees to seal holes in their honeycombs, to smooth out the beehive’s internal walls and to protect the entrance against intruders. Honeybees (*Apismellifera* L.) collect the resin from cracks in plant barks and leaf buds. They masticate the resin and by doing so they add salivary enzymes to it. After this, they mix the partially digested material with beeswax and use it in their hive.

Propolis is a mixture of various amounts of beeswax and resins collected by the honeybee from plants, particularly from flowers and leaf buds. Since it is difficult to observe bees on their foraging trips the exact sources of the resins are usually not known. Bees have been observed scraping the protective resins of flower and leaf buds with their mandibles and then carrying them to the hive like pollen pellets on their hind legs. It can be assumed that in the process of collecting and modeling the resins, they are mixed with some saliva and other secretions of the bees as well as with wax.

Propolis is widely used in Indian folk medicine for the treatment of various illness. The preventive and curative effects of Indian propolis (Propolis samples from Mudivaithanendal, Tamil Nadu) for immunomodulatory activity were evaluated using models of cyclophosphamide treated immunosuppressed rats.

PREPARATION OF EXTRACT

Ethanolic extracts of Propolis (yield = 7.5%) wereprepared by routine methods using rotary vacuum evaporator (Roteva, Equitron, Medica Instruments Mfg. Co, Mumbai) and programmable freeze dryer (Allied Frost, Mumbai) from dried Propolis. Powders of Propolis extracts are light brown color.

Propolis extracts were stored in a refrigerator at 4°C to protect from light anddegeneration, and they are well soluble upto 60mg/mL concentration levels indistilled water used as vehicle as clear light brown solution.

Extraction Procedure

The fresh Propolis were collected and authenticated. ThePropolis was dried in the shade.

Materials

- Rotary Evaporator
- 70% ethanol
- Shade Propolis

Methods

500gm of Propolis extracted with 2liters of 70% Ethanol at 70°C temperature, for 1 hour in a 2 liter round bottom flaskwith condenser attached. Filter and collect the extract. Filter and collect the extract. Theextract was evaporated to drynessunder reduced pressure in a Buchi Rotary Evaporator (Switzerland) at 65°C, to obtain brownish colour residue. This extract was used for the experimentation.

PHARMACOLOGICAL EVALUATION

Experimental animals

The immunomodulatory study was conducted in Swiss albino rats (180-220 g) of either sex. The experimental protocol was approved by the Institutional ethical committee. All animals were housed at 25 ± 5°C in a well-ventilated animal house under 12/12 h light/dark cycle with standard commercial diet as per the ethical guidelines. Animals were acclimatized to the experimental conditions for one week before starting the study to reduce animal stress.

Experimental design

The immunomodulatory activity of Propolis was tested on Cyclophosphamide-treated immune suppressed rat model. The Swiss albino rats were divided into six groups and each group contained 6 animals.

The groups are as follows:

- Group I served as a control received saline solution.
- Group II served as an immuno- suppressant group, received cyclophosphamide at the dosage of (30 mg/kg, i.p.)
- Group III served as a parse control which received Propolis (50mg/kg, i.p).
- Groups IV and V served as the test groups which were immune-suppressed with cyclophosphamide (30 mg/kg, i.p) and treated with Propolis (50 and 100 mg/kg, i.p), respectively.
- Group VI served as the positive control which received cyclophosphamide (30 mg/kg, i.p) along with Standard drug-levamisole hydrochloride (LH) (10 mg/kg bw, i.p).

All the groups treated with Propolis and levamisole hydrochloride were injected on daily basis for 11 days, while cyclophosphamide was given on4,5 and 6^{th} days of the experiment.

At the end of the experiment, the animals were sacrificed by cervical dislocation and the blood was collected using heart puncher in 3% citrate containing tubes. The organs namely liver, spleen, heart and kidney were immediately collected, weighed and stored at 8°C.

Body weight and relative organ weight determination

Body weight and relative organs (Spleen, liver, Heart, Kidney) weight were measured for all animals and the results were expressed as mg of organ weight/g body weight of animal,

Organ weight index= W1/W0 × 100,

where W1 is the weight of Organ and W0 is the weight of body

Histopathological examinations

The liver and spleen tissue slices was dissected and fixed in 10% formalin, embedded in paraffin, sectioned to 4 mm thickness, deparaffinized, rehydrated using standard techniques and finally stained with hematoxylin and eosin. The histological changes in the liver and spleen were examined using light microscopy.

Hematological analysis

The level of WBC, RBC, platelet and heamoglobin level were determined using an automatic cell counter.

Effect of Propolis on cell mediated immune response

Immunization

About 5µg of hepatitis B vaccine (Revac-B, from Bharat Biotech, India) was given as antigen on the 4th day (IM) of the experiment. The vaccine contained aluminum hydroxide as an adjuvant and were preserved with thiomersal.

Cellular immune response

Delayed type Hypersensitivity reaction

The cell mediated immune response was assessed by footpad reaction test. On the 10th day, 5µg of hepatitis B vaccine was injected in the right paw and saline was injected in the left paw. On the 11th day after 24 h, the paw volume was measured using plethysmometer and the results were expressed as % of increase in the paw volume.

Phagocytic response

The phagocytic response was determined according to the method of Wang et al (2012)[127]. On the 7th day of the experiment, the animals were injected with 100

µlof Indian ink via intravenous injection. 50µl of blood was collected with 5µl of 3% citrate by retro-orbital puncher at an interval of 2 and 30 minutes after the injection of ink. Then 25 µl of citrated blood was added to 3 ml of 0.1% sodium carbonate solution to lyse the RBC. The concentration of ink in the blood was read at A675nm using spectrophotometer.

The carbon clearance rate (κ) and phagocytic index (α) were calculated by using the following formula:

Rate of carbon clearance (κ) = $(logOD1 - \log OD2)/(T1 - T2)$

where OD1 is the absorbance at 2 minutes; OD2 is the absorbance at 30 minutes; T1 is the time of blood collection at 2 minutes; T2 is the time of blood collection at 30 minutes

Phagocytic index α =$(\sqrt[3]{k \times A})/(B+C)$

where A is the body weight, B is the liver weight, and C is the spleen weight.

Total antioxidant status of organs

Tissue Homogenate preparation

The liver was homogenized in 50 mM phosphate buffered saline (pH 7.4) by using chilled mortar and pestle at 4°C. The homogenate was centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was used for the determination of antioxidant status of the organs.

Total glutathione level

Estimation of the reduced glutathione (GSH) level was done according to the method of Ellman (1959)[128]. Briefly, 400µl of the tissue homogenate was treated with 400µl of 5% sulphosalicylic acid and mixed well with vortex. Then the mixture was centrifuged at 1000 x g for 10 minutes at 4°C. 100 µl of the supernatant was mixed with 400 µl of 0.3 M phosphate buffer (pH 8.4) and 400 µl of distilled water. Then 100 µl of 0.001 M freshly prepared DTNB (5,5-dithiobis (2-nitrobenzoic acid)) was added and kept in room temperature for 10 minutes. The formation of yellow coloured product was measured at 412nm. The amount of glutathione present in the tissue homogenate was calculated by constructing standard graph with glutathione and the results were expressed as µM/mg of protein.

Lipid peroxidation

The amount of lipid peroxide present in the tissue was estimated according to the method of Stocks and Dormandy (1971)[129]. Briefly, 400 µl of the tissue homogenate was mixed with an equal volume of 10 % Trichloro acetic acid and kept in 4°C for 30 minutes. The proteins were removed by centrifugation at 2000 × g for 10 minutes at 4°C. 500 µl of 1% thiobarbituric acid was added to 500 µl of the supernatent and the mixture was kept in boiling water bath for 30 minutes. The reaction mixture was cooled and centrifuged at 2000 × g for 10 minutes at 4°C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The concentration of lipid peroxide was calculated using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56 x 10-5/M/cm) and the results were expressed in terms of nmoles MDA/mg of protein.

Carbonyl protein

The level of protein damage was determined by carbonyl protein estimation according to the method of Reznick and Packer (1994)[130]. Briefly 200µl of the tissue homogenate was treated with 200µl of 1% trichloro acetic acid and was kept at 4°C for 30 minutes. The mixture was centrifuged at 2000 x g for 15 minutes and the pellet was re-suspended in 10 mM 2,4-dinitrophenylhydrazine in 2N HCl or with 2N HCl as a control blank. This mixture was kept in room temperature for 1 hour and then centrifuged at 2000 x g for 10 minutes. The pellet was washed three times with 1:1 ethanol/ethylacetate solution. Finally, the carbonyl protein containing the pellet was dissolved in 6 M Guanidine. The protein hydrazones were measured at A370 nm using spectrophotometer. The amount of carbonyl protein was calculated from molar extinction coefficient of 22,000 M−1 cm−1 and the results were expressed as µg/mg of protein.

Superoxide Dismutase activity

The level of superoxide dismutase in the tissue was estimated according to the method of McCord and Fridovich (1969)[131]. This method is based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol. Briefly, 100µl of the tissue homogenate was added to 700 µl of 100 mM of Tris-HCl buffer (pH 8.2) containing 30 mM EDTA. Then, 200µl of 2 mM of pyrogallol was added to the solution and measured at 420nm for 60 sec using spectrophotometer. A blank was run without the

addition of homogenate. One unit of SOD activity is the amount of enzyme capable of inhibiting 50% of the rate of autoxidation of pyrogallol compared with the blank and are expressed as units'/mg protein/min.

Catalase activity

The catalase level in the tissue homogenate was estimated according to the method of Sinha (1972)[132] with minor modification. Briefly, add 100 µl of tissue homogenate to 300 µl of 50 mM phosphate buffer pH-7. Then, 100 µl of 200mM H_2O_2 was added to the mixture, mix well and placed it in room temperature for 30 sec. Immediately, after 30 secs, add 500 µl of 1.5% potassium dichromate / acetic acid (weight/volume). The mixture was kept in boiling water bath for 10 minutes and cooled. The absorbance was read at 590 nm against blank using spectrophotometer. The different concentration of H_2O_2 (1-50 µM) was used for construct the standard graph and the catalase activity was calculated from the standard graph of H_2O_2 (1-50 µM) and results were expressed as µmole of H_2O_2 consumed/ mg of protein/ minute.

RESULTS AND DISCUSSION

The immune system plays an important role in defense mechanism and protects the body against various antigens and infectious diseases. The homeostatic balance is maintained by the stimulation or suppression of immune cells and it keeps the body in normal healthy condition. Thus, the immune modulator plays a vital role in maintaining the immune system.

In this study, the immunomodulatory activity of purified Propolis was studied on cyclophosphamide (CYP) treated immuno-suppressed Swiss albino rats. The immunomodulatory activity of Propolis was compared with standard immune activating drug levamisole.

The CYP is an alkylating drug that belongs to the subclass of nitrogen mustard. It is commonly given as a chemotherapeutic drug for cancer treatment and as an immunosuppressant for organ transplantation and autoimmune disorder (Moore 1991)[133]. CYP also causes some side effects such as myelosuppression, immune suppression and oxidative stress which may be life threatening (Wang et al 2011)[134]. The inactive form of CYP is activated by the liver enzyme cytochrome P450 to 4-hydroxycyclophosphamide which transferred to other organs as well. Then, 4-hydroxycyclophosphamide is further converted to phosphoramide mustard and acrolein. The phosphoramide mustard causes cytotoxic damage to cells and acrolein causes some side effects (Sun & Peng 2008)[135].

In this study, CYP is given to the Swiss albino rats to suppress the immune system and induce oxidative stress. The cyclophosphamide effect is expected to reduce the activity of hematological parameters, cell mediated immune responses and macrophage production. Moreover, CYP impaired the organs through its toxic metabolites and caused oxidative stress. The effect of Propolison CYP induced immune-toxicity was examined through myelosuppression, immune suppression and oxidative damage.

Table No. 2 Effect on Propolis on Haemoglobin level

Groups	Haemoglobin (g/dl)
G1	13.40 ± 0.30
G2	10.90 ± 0.22*a
G3	12.85 ± 0.28*b
G4	12.90 ± 0.26*b
G5	13.05 ± 0.29*b
G6	13.20 ± 0.28*b

*a = P < 0.05 compared with normal control

*b = P < 0.05 compared with negative control

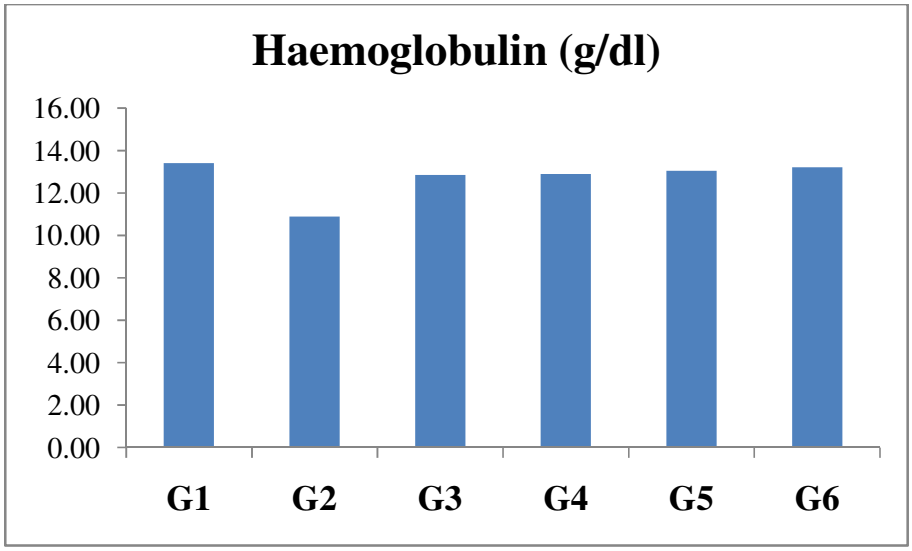


Fig. 9 Effect of Propolis on hematopoietic function against cyclophosphamide -treated rats. G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3-Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5- 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP. Values are expressed as the mean ± S.D. for n = 6 Significance was determined by one-way analysis of ANOVA followed by post-hoc analysis using Newmann Keul's multiple range tests

Table No. 3 Effect on Propolis on RBC level

Groups	RBC (10^6/µL)
G1	7.85 ± 0.16
G2	6.72 ± 0.08*a
G3	8.10 ± 0.12*b
G4	7.60 ± 0.10*b
G5	7.55 ± 0.14*b
G6	7.75 ± 0.10*b

*a = P < 0.05 compared with normal control

*b = P < 0.05 compared with negative control

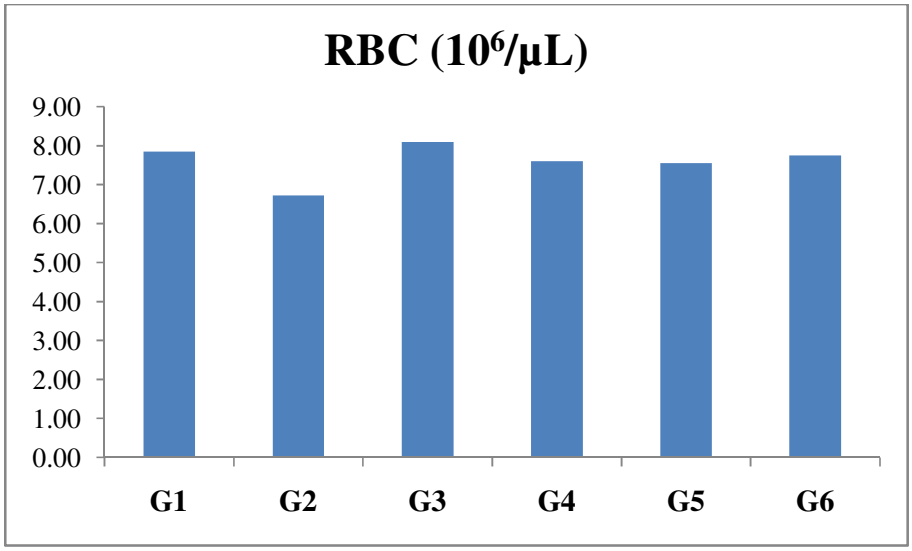


Fig. 10 Effect of Propolis on hematopoietic function against cyclophosphamide - treated rats.G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3- Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5- 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP. Values are expressed as the mean ± S.D. for n = 6 Significance was determined by one-way analysis of ANOVA followed by post-hoc analysis using Newmann Keul's multiple range tests

Table No. 4 Effect on Propolis on WBC level

Groups	WBC (10^6/mL)
G1	7.30 ± 0.22
G2	3.45 ± 0.15*a
G3	6.55 ± 0.18*b
G4	6.90 ± 0.20*b
G5	7.05 ± 0.21*b
G6	6.80 ± 0.19*b

*a = P < 0.05 compared with normal control

*b = P < 0.05 compared with negative control

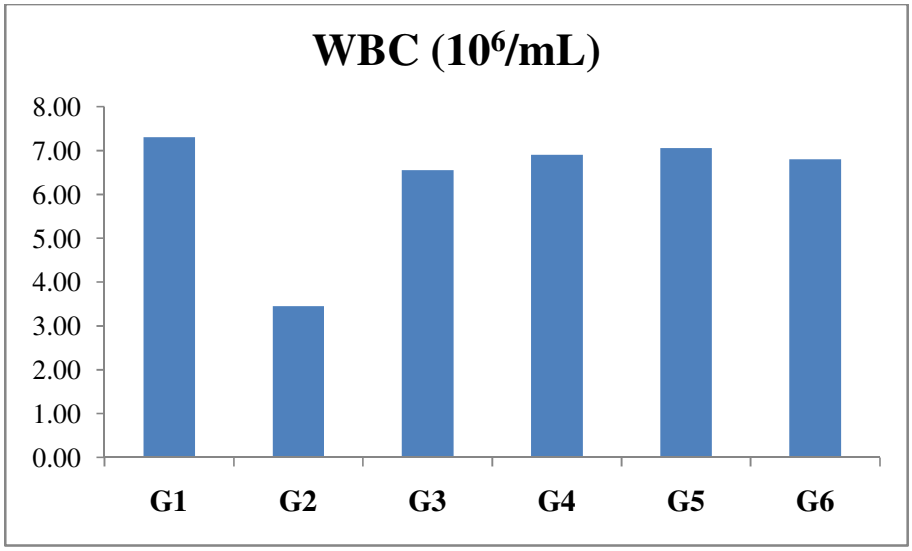


Fig. 11 Effect of Propolis on hematopoietic function against cyclophosphamide - treated rats.G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3- Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5- 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP. Values are expressed as the mean ± S.D. for n = 6 Significance was determined by one-way analysis of ANOVA followed by post-hoc analysis using Newmann Keul's multiple range tests

Table No. 5 Effect on Propolis on Platelets level

Groups	Platelets count (10^3/µL)
G1	840.40 ± 10.50
G2	560.90 ± 6.30*a
G3	830.10 ± 9.60*b
G4	790.20 ± 8.30*b
G5	805.30 ± 8.50*b
G6	802.60 ± 7.90*b

*a = P < 0.05 compared with normal control

*b = P < 0.05 compared with negative control

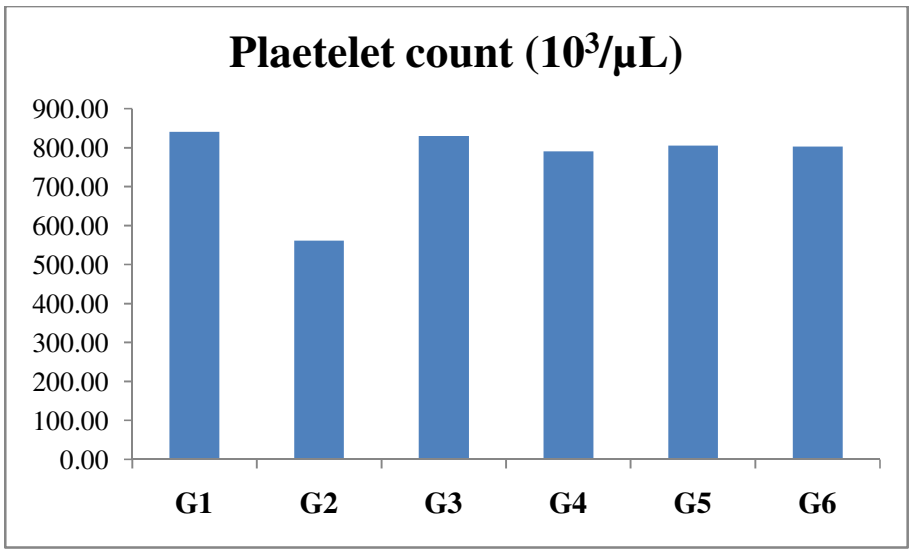


Fig. 12 Effect of Propolis on hematopoietic function against cyclophosphamide - treated rats G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3- Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5- 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP. Values are expressed as the mean ± S.D. for n = 6 Significance was determined by one-way analysis of ANOVA followed by post-hoc analysis using Newmann Keul's multiple range tests

Effect of Propolis on hematopoietic function against cyclophosphamide induced immune-toxicity

The protective effect of Propolis on hematopoietic function against CYP induced immune-toxicity was evaluated by counting the level of hematological parameters like (Haemoglobin) Hb, RBC, WBC and platelet cells. The results of hematological analysis showed that the level of Hb, RBC, WBC and platelet cells were significantly reduced in negative control group of animals (G2- CYP alone treated) when compared to normal control group of animals (G1-Saline alone) ($P<0.01$) (Fig. 9,10,11,12). However, these levels were raised significantly in Propolis treated group of animals (G4 =50 (mg/kg b.w.), and G5 = 100 (mg/kg b.w.) in a dose dependent manner. The increase in the levels of hematological parameters in group of animals of G4 and G5were significant with the negative control group of animals (G2) ($P<0.01$). The levels of hematological parameters in Propolis50 (mg/kg b.w.) alone treated group of animals (G3) was on par normal control group of animals (G1). The hematological parameters in positive control groups of animals (G6) which are treated with standard drug levamisole (10mg/kg) showed significant recovery when compared to negative control group of animals (G2) ($P<0.05$).

The alkylating nature of the CYP, alkylates the DNA and interferes in the synthesis and proliferation of hematopoitic cells leading to myelosuppression. Myelosuppression is the process of decreasing the production of immune cells (leukocytes), oxygen carrying cells (erythrocytes) and the cells responsible for blood clot (thrombocytes) (Urabe 2003)[136]. The results of this study showed that the groups treated with Propolis have improved the production of hematopoietic cells like RBC, WBC, platelets and hemoglobin. The hematopoietic stem cells possess multipotentiality and have the capacity to renew the hematological parameters such as RBC, WBC and platelets. The increase in levels of Hb, RBC, WBC and platelets might have taken place due to induction of hematopoietic stem cells by Propolis. Byon et al (2008)[137] and Frenette and Weiss (2000)[138] have reported induction of mobilization of hematopoietic progenitor cells and boost the immunity by fucoidan from brown algae. The hematological results reveal that the Propolis has protective effect against CYP induced myelosuppression.

Effect of Propolis on cell mediated immunity against cyclophosphamide -treated immunosuppressed rats

The cell mediated immune response is induced by T lymphocytes and their products (lymphokines). These cells are involved in the effector mechanism which provides defense against the infectious organisms, foreign grafts, cancer cells and are also involved in delayed type hypersensitivity reaction (Miller & Peacock 1991)[139]. The cell mediated immune response was determined by delayed type hypersensitivity reaction (DTH) by measuring their footpad thickness. 36% decrease in the paw volume of negative control group of animals (G2) was observed when compared to the normal control group of animals (G1) ($P< 0.05$). The thickness of footpad increased significantly in the groups (G4 and G5) treated with Propolis in a dose dependent manner in comparison with negative group of animals (G2). The footpad volume of 50 (mg/kg b.w.) (G4) and 100 (mg/kg b.w.) (G5) treated group of animals were 30% and 43% higher, respectively ($P < 0.01$ in every group), when compared to negative control group of animals (G2). The footpad volume of 100 (mg/kg b.w) (G5) treated group of animals showed 11% higher than that of normal control group of animals (G1) ($P< 0.01$). The levamisole treated group also showed similar results (i.e) 36% higher footpad volume than control group ($P<0.01$). The group of animals treated with Propolis alone (G3) showed 33% higher footpad volume than the normal control group of animals. The above results also supported by Kim and Joo (2008)[140] who demonstrated cytotoxic T cells enhancing property of fucoidan from C. okamuranus. Wang et al (1994)[141] also reported immune boosting property of fucoidan from L. japonica on immune suppressed mice by activating the macrophage and T- lymphocyte. In this study, the Propolis has enhance the cell mediated immune response through the activation of T-cell.

Effect of Propolis on phagocytic response against cyclophosphamide -treated immunosuppressed rats

Phagocytosis is a process by which immune system effectively remove or engulf microorganisms, cancer cells, inorganic particles and tissue debris. The phagocytic test is used to evaluate the non- specific immunity of the system. The immune cells involved in phagocytosis are called phagocytes (Miller & Peacock 1991)[139]. The macrophages are the major phagocytic cells (neutrophils, monocytes and macrophages). The phagocytic index is calculated from the rate of clearance of

colloidal carbon particles from the circulatory system. The phagocytic index (α) of CYP treated group of animals showed significant decrease (39%) when compared to normal control group of animals (P< 0.05). It indicates that the effect of CYP impaired the immune system and suppressed the levels of phagocytes. The Propolis significantly restored the level of phagocytic index when compared with negative control group of animals (G2) (P< 0.05 with each group). The Propolis at the concentration of 50 (mg/kg b.w), 100 (mg/kg b.w) increased the phagocytic index up to 11% and 29% when compared to negative control group of animals (G2). Moreover, the 50mg/kg Propolis treated groups showed 8% elevated phagocytic activity (P<0.05) than the control group. The standard drug levamisole showed 11.5% increased phagocytic activity than the normal control group. Propolis 4 times higher than that of normal control group at a dose 50mg/kg (Chen et al 2012)[142]. Several authors reported that fucoidan from brown algae have the potency to activate and proliferate the phagocytic system (Song et al 2000; Teruya et al 2009; Wang et al 1994; Yang et al 1995)[143-146]. The results carbon clearance test indicates that Propolis can enhance non-specific immune response against CYP induced immunosuppression.

Effect of Propolis on organ weight index against cyclophosphamide -treated immunosuppressed rats:

The weight index of organs like spleen, liver, kidney and heart reflects the health of the organism. The toxic metabolite produced from CYP is initially metabolized in liver and produces toxic metabolite which is further transferred to other organs. The toxic metabolites primarily impair the immune organs such as liver, spleen and merely affect kidney and heart (Sun & Peng 2008)[135]. In the present study, it was showed that the weight index of the organs like spleen and liver involved in the immune system was significantly reduced in CYP alone treated group of animals (G2) compared to the normal control group of animals (G1) (P<0.05). The weight index reduction of organs like kidney and heart is less when compared to the weight index reduction of liver and spleen. Kanno et al (2009)[147] reported that CYP impose higher impact on the reduction of weight index of liver than on other organs like kidney, heart and lungs. These findings integrate with the results of the present study. The groups of animals given with Propolis alone (G3) did not make much difference on the organ weight. The treatment with Propolis (G4 and G5) improved the spleen and

liver weight significantly compared to negative control group of animals (G2) ($P<0.05$). The rate of index of organ weight recovery was based on their concentration. The results of organ weight index showed that the suppressed health due to CYP induced oxidative stress have recovered to normal after Propolis treatment.

Effect of Propolis on antioxidant status of organs against cyclophosphamide induced oxidative stress

The antioxidant status acts in connection with many diseases and cause immune dysfunction. The drugs like cisplatin, cyclophosphamide or corticosteroids used for immunosuppression generates free radicles (reactive oxygen species) and toxic metabolites (Gate et al 1999)[148]. The imbalance between the reactive oxygen species and antioxidant defense mechanism causes oxidative stress exhibiting inflammatory response and the resulting tissue and cell injury (Cotran et al 1999)[149]. The increased ROS generation will damage macromolecules like lipid, DNA and protein in the tissue. (Colvin 1999)[150]. The immunomodulator from plant origin have the capacity to reduce the oxidative stress through antioxidant mechanism (Joharapurkar et al 2004)[151]. Moreover, Propolis has shown comparably good superoxide radical scavenging capacity and other antioxidant activities. The effect of Propolis against oxidative stress caused by CYP treatment was assessed by the determination of enzymatic and non-enzymatic antioxidant status and levels of macromolecular damage.

Effect of Propolis on non-enzymatic antioxidant status of liver organ

The non-enzymatic antioxidant status plays a major role in maintaining the innate antioxidant status (Williams & Burk 1990)[152]. Glutathione belongs to non-enzymatic antioxidant and also repairs the immunological and neurodegenerative disorders (Raghavendra & Kulkarni 2001)[153]. Reduced glutathione level is a suitable indicator for overall antioxidant defense which maintains alpha-tocopherol and ascorbic acid and is also a coenzyme for glutathione S-transferases and glutathione peroxidases(Jones 2002)[154].

The level of glutathione was significantly reduced in liver of negative control group of animals (G2) (liver= 20.50±1.05) compared to the normal control group of animals ($P<0.05$) (liver = 36.60±1.45). The Propolis treatment on CYP intoxicated

group of animals (G4 and G5) directly increased the glutathione levels significantly based on the dose concentration in liver organs.

Effect of Propolis on enzymatic antioxidant status of organ

The groups of enzymes which are involved in the conversion of active oxygen molecule into non-toxic molecules are superoxide dismutase (SOD) and catalase (CAT). These enzymes maintain the enzymatic antioxidant status of tissue. The antioxidant enzyme superoxide dismutase coverts the superoxide to water peroxide whereas catalase converts hydrogen peroxide to water and oxygen. They are mainly located in peroxisomes, cytoplasm and mitochondria (Halliwell et al 1999)[155]. In this study, the levels of CAT significantly reduced in liver of negative control group of animals (G2) (liver = 1.36±0.24 (U/mg/min) compared to the normal control group (G1) (liver = 4.95±0.35 (U/mg/min) ($P< 0.05$). The Propolis treatment to animal (G4 and G5) significantly restored the decreased CAT level to an equal or above the CAT levels of normal control group of animals in a dose-dependent manner ($P < 0.05$). (G6) of levamisole treated group of animals which are intoxicated with CYP showed 7.2% higher CAT levels than the normal control group of animals (G1). The CAT levels in liver of Propolis alone treated group of animals (G3) was found high by 12.8 % than that normal control group of animals (G1).

Similarly, SOD levels were significantly reduced in liver negative control group of animals (G2) (liver = 40.30±2.60 (U/mg/min) when compared to the normal control group of animal (G1) (liver = 64.30±3.85 (U/mg/min) ($P<0.05$). Like that of CAT, the level of SOD significantly increased upon the dose dependent manner treatment with Propolis and attained the level of normal control group of animals at the concentration of 100mg/kg ($P< 0.05$). Similar to CAT, the Propolis alone treated group of animals (G3) did not show prominent change in the levels of SOD in liver tissues showed 22% higher SOD level than the normal control group of animals (G1).

Table No. 6 Effect of Propolis on enzymatic and non-enzymatic antioxidant status of liver tissue

GROUPS	Catalase U/mg of protein	SOD U/mg of protein	Glutathione U/mg of protein	MDA M/mg of protein	PCO ηM/mg of protein
G1	4.95±0.35	64.30±3.85	36.60±1.45	7.35±0.40	3.20±0.15
G2	1.36±0.24*a	40.30±2.60*a	20.50±1.05*a	14.80±0.85*a	6.55±0.28*a
G3	3.85±0.30*b	56.22±2.90*b	32.40±1.30*b	12.60±0.65*b	5.40±0.18*b
G4	3.60±0.28*b	56.60±2.95*b	30.25±1.10*b	12.80±0.70*b	5.65±0.20*b
G5	4.05±032*b	59.30±3.10*b	33.55±1.38*b	13.30±0.74*b	5.90±0.22*b
G6	4.25±0.34*b	61.20±3.25*b	33.80±1.40*b	13.80±0.80*b	6.10±0.25*b

*a = $P < 0.05$ compared with normal control

*b = $P < 0.05$ compared with negative control

G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3-Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5- 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP. Values are expressed as the mean ± S.D. for n = 6 Significance was determined by one-way analysis of ANOVA followed by post-hoc analysis using Newmann Keul's multiple range tests

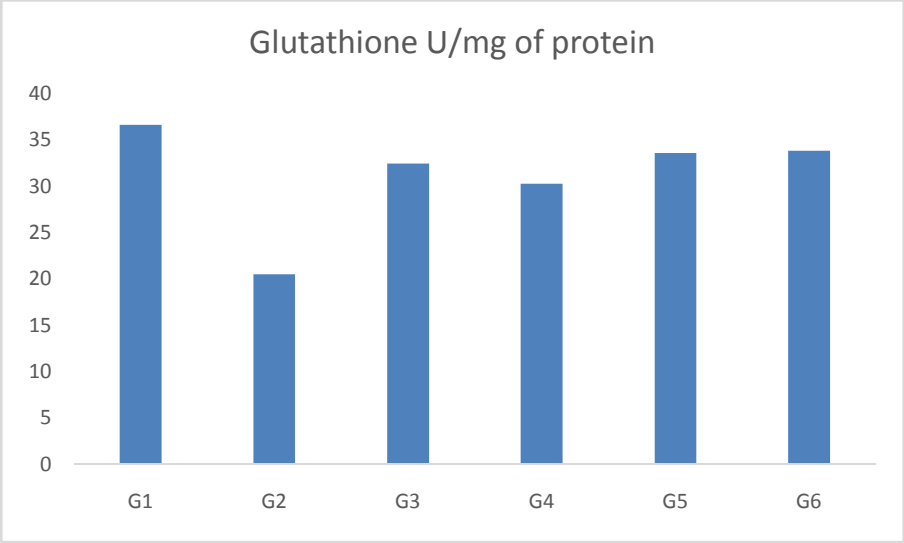


Fig. 13 Effect of Propolis on the level of GSH in organs against cyclophosphamide induced oxidative stress on immunosuppressed rats

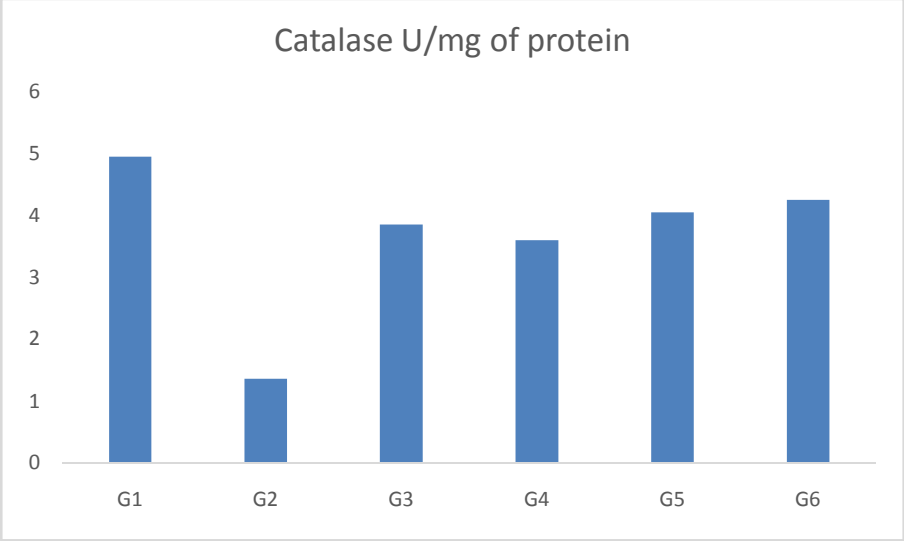


Fig. 14 Effect of Propolis on the level of catalase in organs against cyclophosphamide induced oxidative stress on immunosuppressed rats.

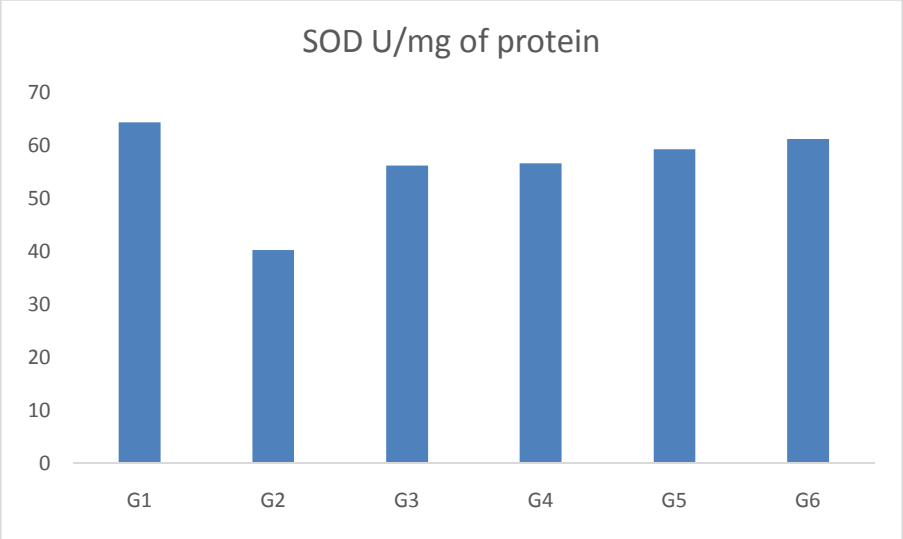


Fig. 15 Effect of Propolis on the level of superoxide dismutase in organs against cyclophosphamide induced oxidative stress on immunosuppressed rats.

Effect of Propolis on macromolecular damage of organ

The important markers of oxidative stress are macromolecular like lipid and protein damage which produce lipid peroxide (MDA) and carbonyl protein (PCO) (Levine et al 1990; Reddy & Lokesh 1992)[157,158]. The MDA and PCO levels were found to be significantly increased in all the organs of the negative control group of animals (G2) compared to normal control group of animals (G1). The increase levels of MDA and PCO indicate that toxic metabolite produced from CYP induced oxidative stress damage the lipids and proteins present in the organ tissue. liver of negative control group of animals (G2) (liver= 14.80±0.85 (ηM/mg of protein)) showed almost 1.5- 2.2 times higher MDA level than that of the normal control group (G1) ($P < 0.05$) (liver = 6.55±0.28 (ηM/mg of protein)). The levels of MDA were found decreased significantly in Propolis treated group of animals (G4 and G5) in dose dependent manner compared to negative control group, G2($P < 0.05$).

Similarly, PCO levels significantly increased 1.5-2 times higher in liver of negative control group of animals (G2) (11.27±0.64 (µg/mg of protein), 10.49±0.52 (µg/mg of protein), 7.79±0.31(µg/mg of protein)) compared to the normal control group of animals (G1) (6.87±0.43 (µg/mg of protein), 5.25±0.37 (µg/mg of protein), 5.00±0.17 (µg/mg of protein). The increased levels of PCO on CYP toxicity decreased significantly on Propolis treatment in a dose dependent fashion (G4 and G5) ($P<0.05$).

The CYP toxicity induced high MDA and PCO levels were found normal at concentration of 100 (mg/kg b.w.) and 10 (mg/kg b.w) of Propolis and levamisole, respectively. Kim et al (2014)[159] also reported that fucoidan significantly reduced MDA levels against 2,2-azobis dihydrochloride induced oxidative stress in zebrafish model. The fucoidan from C. okamuranus also reduce the MDA levels in Sparague-Dowley (SD) rats (Thomes et al 2010)[160].

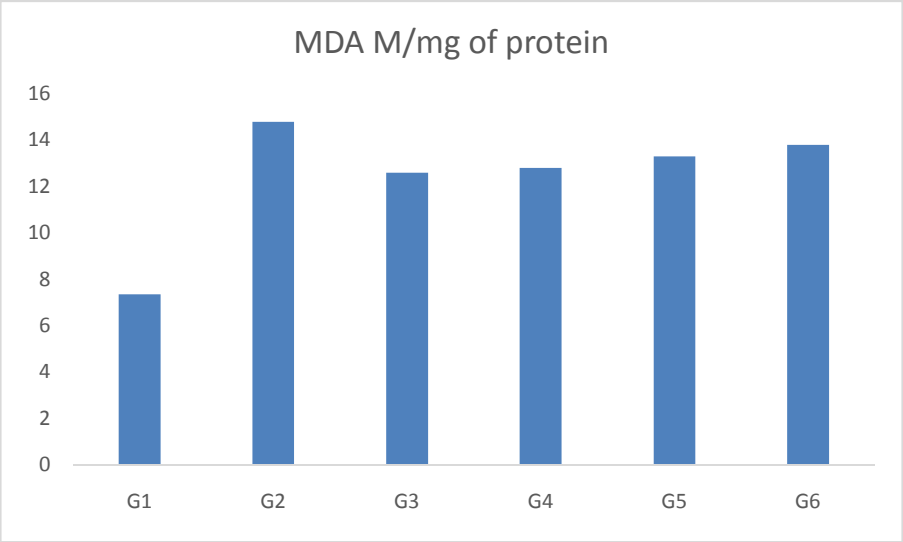


Fig. 16 Effect of Propolis on the level of MDA in organs against cyclophosphamide induced oxidative stress on immunosuppressed rats:

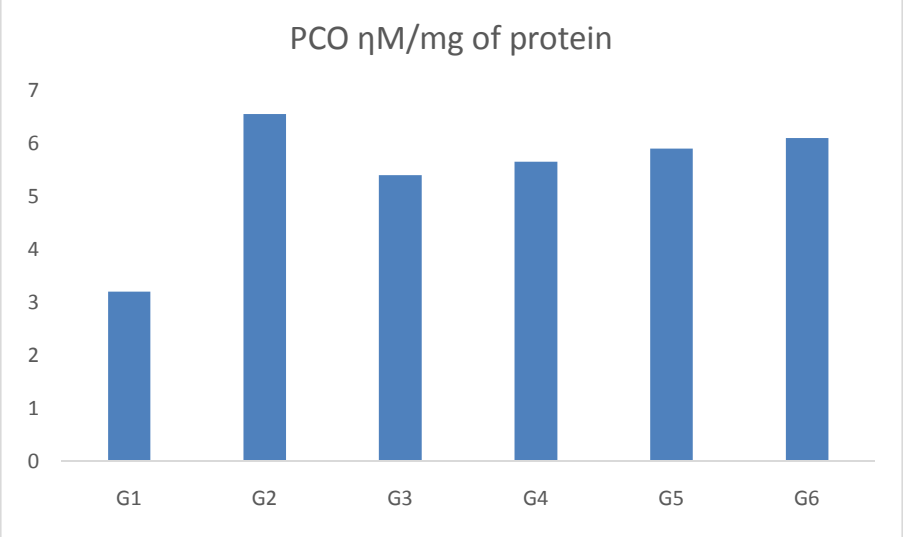


Fig. 17 Effect of Propolis on the level of carbonyl protein of organs against cyclophosphamide induced oxidative stress on immunosuppressed rats:

HISTOPATHOLOGICAL ANALYSIS

The morphological analysis of organs is studied further to investigate the effect of Propolis on the structural damage of organ due to CYP induced toxicity. The toxic metabolite produced by CYP is metabolized in liver and it transfers the reactive metabolites to the spleen.

The Histopathological analysis of liver section of CYP treated animal showed white pulp and congested sinusoids and also showed toxic change like inflammation. The toxic change effected by CYP induced toxicity was gradually decreased upon treatment of Propolis (G4- 50 (mg/kg b.w.) and G5-100 (mg/kg b.w.). The Propolis alone treated group of animals (G3) did not show any characteristic change in the liver section compared to normal control group of animals (G1) (Fig. 18).

The present study showed that the purified Propolis provide protection from oxidative stress triggered by CYP and recover the damage. CYP treatment significantly elevated the levels of macromolecule damage (MDA and PCO) and decrease the levels of enzymatic (SOD and CAT), non-enzymatic (GSH) antioxidant status in Liver. At the same time, Propolis treatment at the concentration of 100mg/kg b.w. reverse the effect of CYP induced damage via increasing CAT, SOD and GSH levels and thus protecting macromolecular damage and decrease MDA and PCO level. The morphological analysis also supports above study and shows the ability of Propolis to repair the damage caused by CYP induced oxidative stress.

G1 section showed normal liver parenchyma with central vein and hepatocytes radiating from it; G2 section showed toxic change in liver due to CYP toxicity with portal triad showing mild inflammation; G3 section showed normal liver parenchyma with central vein and hepatocytes radiating from it; G4 and G5sections showed liver parenchyma with hepatocytes with slight, moderate and marked recovery respectively from CYP induced damage; G6 section showed liver parenchyma with central vein and hepatocytes exhibiting significant recovery from toxic change of CYP.

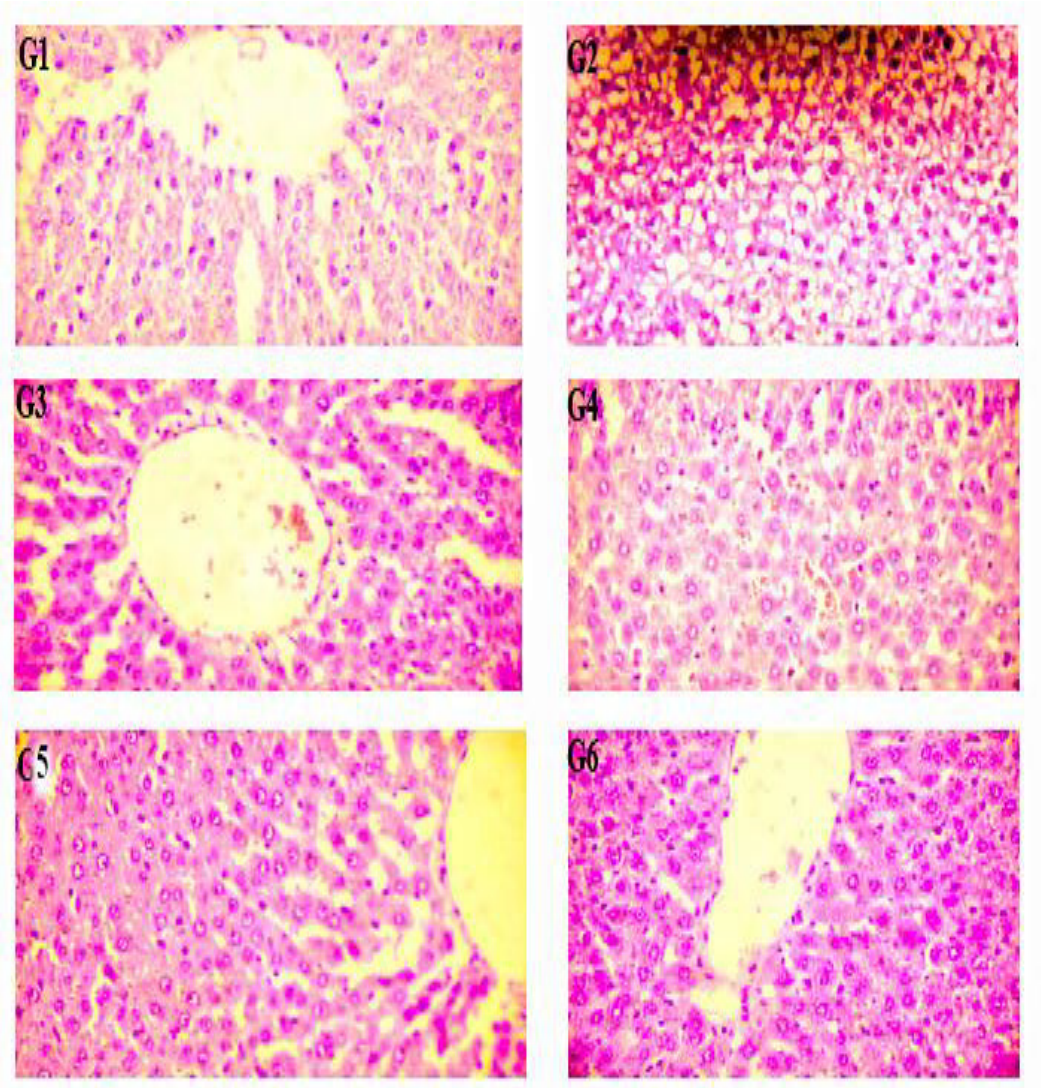


Fig. 18 Effect of Propolis on histopathological changes in the liver tissues against cyclophosphamide induced damage

G1-normal control; G2-Negative control (30mg/kg.b.w. CYP alone treated); G3-Perse control (50 mg/kg b.w. Propolis alone); G4- 50 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G5 - 100 mg/kg b.w. Propolis + 30mg/kg b.w. CYP; G6-10 mg/kg b.w. Levamisole+ 30mg/kg b.w. CYP.

CONCLUSION

In conclusion, the present study has proved that Propolis not only enhance the immune system but also protect the organs against oxidative stress. The results of the present study recommend that Propolis could be a prominent natural immunomodulating molecule with therapeutic value.

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